PHILIP MORRIS U. S. A.

INTER-OFFICE CORRESPONDENCE

RICHMOND, VIRGINIA

CONFIDENTIAL

To: Dr. T. S. Osdene

Date: November 4, 1981

From: J. L. Charles

Subject: Plans and Objectives for 1982--Biochemical Research Division

The Plans and Objectives prepared by the individual project leaders in the Biochemical Research Division are attached. Each project leader prepared his/her report after receiving input from his colleagues and meeting with the manager, technical coordinator, and other project leaders so that project plans are consistent with overall plans and objectives of the division.

The objectives are:

1. Develop an integrated program for control of insects which infest stored tobacco, processed filler and finished cigarettes.

2. Develop methods for the collection, isolation, identification and quantitation of tobacco and cigarette components which affect the *in vivo* and/or *in vitro** bioactivity of smoke products.

3. Establish a matrix or battery of *in vitro* bioassays for the evaluation of the biological effects of new cigarette products, additives, and smoke products.

4. Conduct research investigations to determine the factors responsible for the biological activity of smoke products in each assay in the battery.

An overview of the plans in their logical progression is presented below.

*_in vivo_ means biological studies conducted in whole animals (mammals).

in vitro means biological studies conducted in culture.

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CHARGE NUMBER 1101--ENTOMOLOGICAL RESEARCH

Cigarette beetle physiological studies will be continued. The continuing trend to eliminate the use of highly toxic pesticides as control agents dictates a continued effort in this area. Research in this area will consist of: (1) the effect of temperature and relative humidity on beetle growth and survival; (2) the evaluation of azadirachtin from neem seeds as an insect feeding inhibitor; (3) the evaluation of serricornin, a synthetic sex pheromone, as a trap bait for primary and manufacturing areas; (4) investigation of prism projected rays as a light attractant; (5) studies of the adult beetle flight and response behaviors; (6) development of a screening technique for inhibitors of the larval midgut symbiont, and (7) the study of NALED (a low toxicity organophosphate) as a sex pheromone beetle trap toxicant.

The research program associated with the "commercial trial" of KABAT®, 5% methoprene in ethanol, will continue. A study of bright tobacco treated with 5 ppm methoprene was reinitiated in November, 1980 and will continue through November, 1982. The Maury Street, Chesapeake and Cabarrus warehouses storing only KABAT® treated tobacco will be monitored by checking beetle trap counts. We will continue to support the stemmeries in KABAT® application throughout PM USA and PMI facilities as requested.

Upon request, we will continue to provide consultation and technical services on insect control problems for other Departments within Philip Morris Incorporated. These services will consist of on-site inspections for existing or potential infestation of stored or processed tobacco, providing insects and insect counts for control tests and providing updated information on state and federal regulations pertaining to the use of pesticides. Long range monitoring studies involving steam or steam-vacuum insect killing efficacy checks will be continued.

CHARGE NUMBER 6910--CONDENSATE PREPARATION, PROCESSING, AND PYROLYSIS STUDIES

The collection and processing of cigarette smoke condensate and other smoke products is crucial since these products are starting materials for most of the other projects in the division. An outside study beginning in the first quarter of 1982 will require Elmenhorst cold trapped condensate, preparation of acetone solutions of processed condensate, and quality assurance determinations of nicotine and water in condensate. This is a high priority program requiring a continuing effort throughout the year.

Mainstream smoke will continue to be collected for *in vitro* and chemical testing. Various methods used for collection of condensate and/or smoke volatiles include impaction trapping, solvent filled impaction trapping, Elmenhorst cold trapping, Cambridge filter pad and volatiles or gas phase collection techniques.

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Sidestream cigarette smoke (SS) collection methods will continue to be developed and assume increasing importance. Current methods involve smoking a single cigarette at a time and methods will be investigated to allow simultaneous smoking of four cigarettes in a sidestream smoke chamber for small scale *in vitro* and chemical testing. To support sidestream smoke condensate fractionation studies, we will initiate studies on methods to collect sidestream smoke from a 30-port Borgwaldt smoking machine.

As with mainstream smoke, methods for collecting sidestream smoke will include impaction trapping, vapor phase cold trapping, solvent filled impaction trapping, and Cambridge pad collection. These methods will be used to support chemical and *in vitro* studies. We will continue to evaluate Cambridge pad collected SS directly extracted with dimethylsulfoxide (DMSO) in screw capped vials to facilitate direct MS-SS comparisons on the same cigarette and avoid extraction of pads in a fritted funnel.

Trapping of SS volatile and nonvolatile nitrosamines in gas washing bottles will continue to be investigated. Methods will be devised in collaboration with 6908 to introduce nitrogen into SS at varied flow rates in an effort to reduce artifactual formation of volatile nitrosamines.

The small Mellen furnace with a quartz pyrolysis tube allows pyrolysis of filler at temperatures up to 900°C. This method will continue to be used in the investigation of the effects of pyrolysis conditions on the *in vitro* biological activity produced from pyrolysis of a number of fillers and blends. Pyrolyzate base fractions will be prepared for *Salmonella*/microsome testing and chemical profiling. Filler treatment with potassium salts or nicotine prior to pyrolysis and the effects on chemical profiling and *in vitro* activity will be investigated.

Nitrogen-free LTF-5E control sheet and LTF-5E plus various additives will be prepared on an "as needed" basis. The sheet material will be shredded for filler, made into cigarettes and smoked for chemical and biological studies.

CHARGE NUMBER 6908--CHEMICAL STUDIES OF CONDENSATE

Chemical studies of condensate will continue to focus on the isolation, identification, and quantitation of compounds thought to be relevant to the *in vitro* and *in vivo* biological activity of smoke products. This effort requires a continual monitoring of the literature related to biologically active smoke components.

Both volatile and nonvolatile nitrosamines will be determined in sidestream smoke with methods development in conjunction with 6910. Filler and MS smoke nonvolatile nitrosamines will be determined on a series of model cigarettes with the aim of establishing a data base. Nitrosamine determination will continue on a request basis for Project 6900.

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Burley CSC base fraction studies, in conjunction with 6906 will continue. Major emphasis will be directed toward HPLC separation of the (*Salmonella*) active LH-20 fractions from the toluene/50% aqueous methanol partition step carried out on burley base fraction pot residue. Subfractions with significant activity will be further separated with isolation/identification of active compounds as a goal.

Evidence for the presence of Glu-P-1/Glu-P-2 (pyrolysis products of glutamic acid) has been obtained on an LH-20 fraction of burley base fraction and isolation and NMR confirmation will be attempted.

The feasibility of concentrating *Salmonella*/microsome activity of burley base fraction and subfractions by preparative GPC will be studied.

The effect of nicotine on *in vitro* activity will be studied in collaboration with 6902, 6904, 6906, and 6910. Nicotine at several levels will be added directly to the pot residue from burley and bright base fractions and tested by *in vitro* assays to determine if a direct effect of nicotine can be demonstrated. Similar studies will be carried out on CSC and acid/neutral fractions from burley and bright tobacco cigarettes. A comparative study will then be conducted on bright and burley cigarettes with varying levels of added nicotine to determine the relationship, if any, between nicotine, nicotine pyrolysis products and *in vitro* biological activity. In addition a nicotine aza-arene study will be initiated with nicotine added to LTF-5E followed by an isolation of nicotine derived (or not) aza-arene fraction. Using gc, gc/ms, and the *Salmonella*/microsome assay, an attempt will be made to correlate activity with individual components. Identification of these active components will follow.

Work will begin on a HPLC/*in vitro* interface with 6902 and 6906 with the goal of determining specific activity of each peak in an HPLC chromatogram using the *Salmonella*/microsome or the 8-azaguanine forward mutation assay.

Interactive effects in which inhibitory compounds are masking the *in vitro* effects of various fractions have been demonstrated and will be investigated further.

Research proposals will be developed for microsome-independent activity (MIA), electrophiles-glutathione (GSH) interactions and other biologically active compounds--cell--smoke interactions.

Sidestream studies involving nitrosamines, fractionation and chemical characterization of SS fractions will continue or be initiated.

The method for HPLC analysis of carbonyls in VP will be reestablished and updated to be used in a collaborative study with 6904 using the L5178Y assay.

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A collaborative study with 6902 on the D_4 yeast strain and activity of acid/neutral and base fractions will be completed. A PAH fraction procedure and capillary gc analysis will be utilized to study a selected number of cigarettes for comparison of *in vivo* activity, *in vitro* activity and PAH profiles.

Work on the thermal decomposition of duvatriene diols and tobacco diterpenes will be completed shortly.

Testing of base fractions from MS and SS smoke derived from RL base web with added KOAc will be completed. The fractions will be analyzed for 2AC and results correlated with *Salmonella*/microsome assay using LTF plus amino acids or protein as the starting material.

After initial installation and training on the VG MM-16 gc/ms/ds, evaluation of various packed and/or capillary columns for base fraction analysis will begin. Evaluation of a fast atom bombardment ion source for nonvolatiles and consideration of desorption chemical ionization to enhance volatility will be pursued.

A Chromatotron has been ordered for use in base fractionation studies of CSC.

CHARGE NUMBER 6906--BIOLOGICAL EFFECTS OF SMOKE

The majority of the *Salmonella*/microsome mutation assay studies will involve a continuing collaborative interaction with 6900, 6908, and 6910 project personnel. These studies are directed towards refining our understanding of the determinants of CSC activity and the reader is referred to the 6906 Plans and Objectives for a detailed treatment.

Studies involved with assay standardization and quality assurance will include transition from the Sigma to the DEC system, preparation of a new TA98 frozen ampoule stock culture and purchase and complete evaluation of a new batch of Aroclor®-1254 induced rat liver microsomes. Aroclor® induced hamster liver microsomes will also be evaluated to broaden our metabolic activation capability. A determined effort will be made to complete the interfacing of the automatic colony counter with the main computer (with CAD personnel) to improve our ability to handle an increased workload.

A research proposal will be written to approach the evaluation of a number of filler and construction parameters on CSC activity (effect of dilution, rod weight, RTD, firmness, cuts per inch, *etc.*, on coal temperature and IT CSC activity).

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In a collaborative study with 6902 and 6904, a research proposal will be developed to address the question of assay sensitivity and specificity when known mutagens are added to CSC and later when known mutagens are added to filler and the resulting smoke condensate is tested in the *Salmonella*/microsome assay (and other assays). This is a crucial study which will aid us in interpreting results obtained on biological activity testing of additives plus tobacco.

CHARGE NUMBER 6904--BIOCHEMICAL METHODS DEVELOPMENT AND UTILIZATION

This project is involved with the development of short-term *in vitro* mammalian cell assays for evaluation of potential biological activity of cigarette smoke products or potential additives.

A difficulty with the L5178Y mouse lymphoma assay has been the inability to obtain a negative response in the assay in our laboratory. Pure chemical compounds known to produce no bioactivity *in vitro* or *in vivo* will be tested. If this is unsuccessful, a redefinition of a negative response will be in order. The MS and SS activity of LTF-5E (nitrogen-free) will be evaluated in the L5178Y assay and filler composition will be altered to manipulate activity.

The V79 Chinese Hamster lung cell assay is being developed using a mutation endpoint. Dose-response relationships with a known direct acting mutagen has been established and will be replicated. A mutagen requiring metabolic activation will be studied next followed by reproducibility studies and additional classes of compounds. This assay is attractive since it has the potential for multi-endpoints. In that regard, efforts are underway to establish the "promotion assay" endpoint of V79 which is based on the apparent inhibition of metabolic cooperation by promoters. The assay will be established with the potent promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Dose-response studies with other pure compounds (known promoters and negative compounds) will follow.

CHARGE NUMBER 6902--BIOCHEMICAL SPECIAL INVESTIGATIONS

The yeast mitotic gene conversion assay using strain D_4 is a continuing effort in this project. This assay suffers from an apparent lack of sensitivity to CSC and fractions. Good dose-response curves have been obtained with CSC but frequently the activity does not exceed two times background. A number of acid/neutral and base fractions are being studied with the specific goal of acquiring sufficient data to make an intelligent decision whether or not to proceed with this assay. Plans for 1982 are dependent on the outcome of that evaluation. Strain D_7 has been added to our collection and will be evaluated in the mitotic gene conversion endpoint for utility in additive testing.

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In 1982, we will establish the *S. typhimurium* forward mutation assay based on 8-azaguanine resistance. This assay is attractive because the genetic target for forward mutation is much larger than the reverse mutation of the Ames reverse mutation assay. Also, the forward mutational event (normal cell is made sick) mimics the real life situation better than reverse mutation (sick cell made normal). So, the combination of decreased specificity coupled with the forward mutation should increase our capability for detecting a mutagen. The type of mutational event (base substitution or frame-shift mutation) can be studied with the Ames tester strains. This assay will require a mini-validation study as part of its establishment.

We also expect to establish the Sister Chromatid Exchange assay in 1982 as a sensitive indicator of chromosomal damage and for monitoring of DNA repair. We will use the Chinese hamster V79 cells for this assay and further utilize the multi-endpoint capability of that cell line. Definitive plans are dependent on filling an open requisition with a qualified person.

The *Tradescantia* stamen hair assay will be studied for feasibility with regard to its potential for use in monitoring SS biological activity in dilute atmospheres.

Finally, the emphasis on new assay development will shift. Previously assays were developed for measuring the biological activity of cigarette smoke condensate; a most complex task certain to require extensive development before useful results were obtained. Only then was an assay used to evaluate potential additives. In this writer's opinion, Philip Morris would be better served if an assay were established with pure compounds and then utilized immediately for testing additives. In this way, the advantage of the assay to PM is immediately apparent. Then one could proceed to the much more complex task of elucidating the biological activity of cigarette smoke condensate and fractions. It is this approach which will establish a *battery* of short-term *in vitro* assays in the shortest possible time.

nwp

cc: Dr. R. N. Ferguson
Dr. M. A. Manzelli
Dr. T. T. Yu
Dr. R. W. McCuen
Mrs. K. Rapp Sherwood
Dr. A. H. Warfield
Mr. R. D. Hellams
Mr. R. D. Carpenter
Ms. J. E. John
Dr. R. A. Pages

Attachments: six

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